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PHARMACOKINETICS OF 4-HYDROXYANDROSTENEDIONE IN MAN AFTER INTRAMUSCULAR INJECTION OF DIFFERENT FORMULATIONS AND THE EFFECT OF THIS DRUG ON PLASMA AROMATIZABLE ANDROGENS AND 17β-ESTRADIOL CONCENTRATIONS

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Summary—Pharmacokinetics of 4-hydroxyandrostenedione (4-OHA), a potent aromatase inhibitor under investigation for treatment of postmenopausal breast cancer, were studied using two formulations with different particle sizes of 4.2 and 8.0 μm, respectively. A single 250 mg dose of 4-OHA of each of the two formulations was administered in two different periods to six healthy male volunteers and blood samples were collected for up to 14 days. 4-OHA plasma levels were determined using the isotope dilution mass spectrometry method. Comparison of the pharmacokinetic profiles of the two formulations did not show any statistically significant difference, even though the 4.2 μm particle size gave apparently higher levels at 24 h. Using this formulation, the effects of 4-OHA on the plasma levels of aromatizable androgens (testosterone and androstenedione) and 17β-estradiol were studied. An isotope dilution mass spectrometry method was developed for the simultaneous quantitative determination of these steroids in human plasma. The analysis of plasma samples showed a significant reduction of plasma estradiol concentrations (50%) which coincided with the maximum concentration peak of the inhibitor, whereas no significant changes in androgen levels were observed.

INTRODUCTION

4-Hydroxy-4-androstene-3,17-dione (4-OHA), a potent aromatase inhibitor, has been under investigation for treatment of postmenopausal breast cancer for the last few years [1, 2]. More recently aromatase inhibitors have also been proposed for treatment of human benign prostatic hypertrophy (BPH) [4, 5]. The kinetics of these drugs in human male plasma are thus of clinical interest.

Unlike some non-steroidal aromatase inhibitors, 4-OHA does not have any relevant effect on adrenal steroidogenesis [6]. When administered to postmenopausal women, the compound is without significant side effects except for occasional sterile abscesses at the injection site [1]. This is thought to be due to local effects of the steroidal material. The evaluation of the optimal particle size which can realize the highest plasma concentrations of the drug versus the lowest incidence of side effects is, therefore, of clinical relevance. The injected dose should also be important and consequently during the last years the initial dose of 500 mg every 2 weeks was reduced to 250 mg [6].

In this paper we report the pharmacokinetics of two different formulations of 4-OHA with different particle size (4.2 and 8.0 μm) injected as a 250 mg dose in six healthy male volunteers. Quantitative determinations of 4-OHA plasma levels were obtained using an isotope dilution GC/MS method. In addition the effects on circulating aromatizable androgens (testosterone, androstenedione) and 17β-estradiol (E_2) were studied using the formulation which gave the highest concentration of 4-OHA.

EXPERIMENTAL

Materials

4-OHA was provided by Ciba-Geigy Medical Department, (Origgio, Varese, Italy).

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Testosterone, androstenedione and E_2 were purchased from Sigma. Deuterated standards were previously synthesized in our laboratories [7, 8]. Solvents of analytical grade were purchased from Carlo Erba.

Extrelut columns were obtained from Merck. Sephadex-LH-20 was purchased from Pharmacia. *N*, *O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA), pentafluoropropionic anhydride (PFPA), heptafluorobutyric anhydride (HFBA), and trimethylsilylchloride (TMSCl) were obtained from Fluka.

All analyses were performed using a Hewlett-Packard GC/MS system composed of a 5890 series II gas-chromatograph equipped with a 5971A Mass Spectrometry Detector and a 7673A automatic injector.

Methods

Blood samples

Two different formulations of 4-OHA (particle size 4.2 and 8.0 μm) were injected intramuscularly as a single dose of 250 mg in 6 healthy male volunteers aged 20 to 45 years. Blood samples were collected before (time point 0) and 4, 8, 12, 24, 30, 36, 48, 54, 60, 72, 96, 168, 240 and 336 h after each intramuscular injection. There was a wash-out phase of at least 4 week duration between the administration of the two formulations. Immediately after collection the samples were centrifuged and plasma was stored at -20°C in two aliquots of 2 ml until analysis.

4-OHA determination

4-OHA concentrations were determined using the isotope dilution mass spectrometry method described previously by us [3]. The internal standard was 7,7[^{2}H]$_2$4-OHA (D_2-4-OHA).

Standard solutions. Ethanol solutions of 4-OHA and its deuterated standard were used at the following concentrations (pg/μl): D_2-4-OHA: 200, 4-OHA: 20; 100; 200; 400.

Calibration curve. A six point calibration curve from 0 to 6 ng of 4-OHA was used, with 4 ng of deuterated standard for each point. Each point was obtained by adding the appropriate quantities of 4-OHA and internal standard to a control plasma (200 μl) and performing the same purification and derivatization procedure of the plasma samples. The peak area ratios (PAR) were calculated using the signals at 359.2 and 361.2 m/z for 4-OHA and its deuterated standard, respectively. Over this range of standards the calibration curve was linear (corr. coeff. r ranged from 0.97 to 0.99).

Sample preparation and purification. A solution of the internal standard D_2-4-OHA in ethanol (4 ng in 20 μl) was added to the following volumes of plasma samples: 0.5 ml for samples collected 0, 72, and 96 h after drug injection; 1.0 ml for samples collected 168, 240, and 336 h after drug injection; and 0.2 ml for samples collected 4, 8, 12, 24, 30, 36, 48, 54, and 60 h after drug injection. These volumes were established, after an initial screening on a wide range curve (0–50 ng), to have samples containing between 0 and 6 ng of 4-OHA. 0.18 ml of water were added to the 0.2 ml samples before adding the internal standard solution to prevent protein denaturation. After the addition of internal standard, the plasma samples were equilibrated overnight at 4°C then purified using a solid phase extraction with Merck Extrelut columns (1 or 3 ml) eluted with hexane (10 or 30 ml). The eluted samples were evaporated under N_2 at 40°C.

Derivatization. Immediately after purification, derivatization was performed, treating the residues with 30 μl of BSTFA for 1 h at room temperature to obtain the 4-TMS derivative. The reaction mixtures were then evaporated under N_2 at 40°C and the residues dissolved in 40 μl of heptane.

GC-MS analysis. For sample analysis, the instrument was equipped with an HP1 column (12 m × 0.2 mm × 0.32 μm). 2 μl of the heptane solutions of each sample were automatically injected in split–splitless mode (purge off time 0.75 min). The column temperature program was: 70°C for 1 min then 40°C/min to 220°C, 220°C for 0.5 min then 10°C/min to 300°C, 300°C for 5 min. In these conditions the retention time of 4-OHA was 10.7 min. The two ions monitored by selected ion monitoring (SIM) were at 359.2 and 361.2 m/z for 4-OHA and D_2-4-OHA, respectively, corresponding to the M-15 fragments.

Androgen and estrogen determination

A new isotopic dilution GC/MS method for the simultaneous determination of testosterone (T), androstenedione (A) and E_2 was developed with modifications of the reported method for the determination of E_2 in plasma using isotope dilution mass spectrometry [9]. Because of the limited volume of the samples (2 ml) it was necessary to perform the determination of the three steroids simultaneously. Estrone determination was not performed because we have been unable to find an appropriate derivatization

procedure which will achieve accurate measurement of this steroid at very low concentrations in human plasma samples, and avoiding non-specific interferences.

Internal standards. The following internal standards were used [7, 8], 16,16,17[^{2}H]$_3$ testosterone (D_3-T), 7,7[^{2}H]$_2$ androstenedione (D_2-A),16,16,17[^{2}H]$_3$ estradiol (D_3-E_2).

Stock and working solutions. Stock solutions of analytes and internal standards were prepared in ethanol at a concentration of 1 μg/μl. Working solutions were prepared at a concentration of 10 ng/μl, 1 ng/μl and 10 pg/μl by diluting stock solutions.

Calibration curves. Five point calibration curves were used in the following ranges: 0–20 ng for T, 0–4 ng for A and 0–150 pg for E_2. For each point the following amounts of deuterated standards were added: 5 ng for T, 1.25 ng for A and 150 pg for E_2. No plasma was used for these calibration curves. Each point of the calibration curve was derivatized using the same procedure used for the corresponding fraction of plasma samples, but without the purification procedure. The PAR were calculated using the following signals: 580.4 and 583.4 m/z for T and D_3-T, respectively; 432.3 and 434.4 m/z for A and D_2-A, respectively; and 540.4 and 543.4 m/z for E_2 and D_3-E_2, respectively.

Points fitted with polynomial curves of the second order for androgens, while there was a linear correlation for E_2 (corr. coeff. r ranged from 0.98 to 0.99).

Sample preparation and purification. A solution containing D_3-T and D_2-A (20 μl, respective concentrations: 250 and 62.5 pg/μl), and a solution of D_3-E_2 (15 μl, 10 pg/μl) were added to 2 ml plasma samples.

Samples were equilibrated overnight at 4°C. After equilibration a two steps purification procedure allowed us to separate androgens from estrogens and to perform a more suitable derivatization for each steroid. This procedure includes a first purification step on Extrelut 3 columns followed by a chromatographic separation on Sephadex LH-20 columns. 1 ml of distilled water was added to the equilibrated samples. Samples were then poured into Extrelut 3 columns and left aside for 30 min.

Columns were eluted with 15 ml of dichloromethane containing acetic acid (10 ml/l). After evaporation under N_2 at 40°C, dry residues were dissolved in 200 μl of dichloromethane–methanol–acetic acid (95:5:1, by vol) and applied to the top of Sephadex LH-20 columns (120 × 7 mm) that had been swollen in the same eluent. The same eluent (150 μl) was used to wash the vial and the pipette and was then poured on the column. Fractions were collected with the following pattern: 1 ml discharged; 3 ml collected (fraction containing T and A); 1.5 ml discharged (this fraction contained estrone); 3.5 ml collected (fraction containing E_2). Eluted fractions were evaporated under N_2 at 40°C and dry residues were derivatized.

Derivatization. (1) T and A were derivatized as pentafluoropropionic esters: 3,17-$(PFP)_2$-T and 3-PFP-A, respectively. The derivatization was performed by dissolving the dry residues in 100 μl of acetone and treating with 25 μl of PFPA at 60°C for 1 h. The reaction mixture was evaporated to dryness and the residue dissolved in 40 μl of heptane.

(2) E_2 was derivatized as mixed trimethylsilyl ether-heptafluorobutyric ester (3-TMS-17-HFB-HFB), using a multi-step procedure [9]. The HFB ester 3,17-$(HFB)_2$-E_2 was first prepared with the same procedure used for the PFP esters of T and A, the reaction residue was then dissolved in 80 μl of methanol containing pyridine (100 ml/l) and left aside for 10 min at room temperature. After careful evaporation, the residue was treated with 75 μl of BSTFA containing TMSCl (50 ml/l) for 15 min at room temperature. The reaction mixture was evaporated under N_2 at 40°C and the residue dissolved in 20 μl of heptane.

GC/MS analysis. For these samples the instrument was equipped with an RLS 200 column (15 m × 0.18 mm × 0.25 μm). 3 μl of the heptane solutions were automatically injected in split–splitless mode (purge off time 2.0 min). The column temperature program was: 70°C for 2 min then 40°C/min to 240°C, 240°C for 2 min then 15°C/min to 300°C and 300°C for 10 min. In these conditions, the retention times were: 8.6 min for T, 9.0 min for A and 9.5 min for E_2. The ions monitored in SIM mode were respectively: 580.3 and 583.3 m/z for T and D_3-T, 432.3 and 434.3 m/z for A and D_2-A and 540.4 and 543.4 m/z for E_2 and D_3-E_2. All these ions corresponded to the molecular ions.

Sensitivity

Sensitivity was evaluated using signal/noise ratios (S/N) calculated injecting derivatized

standards. Injection of 0.5 pg of 4-OHA produced a S/N of 5/1; 10 pg of T produced a S/N of 28/1; 10 pg of A produced a S/N of 5/1 and 10 pg of E_2 produced a S/N of 35/1.

Recoveries

The mean recovery for 4-OHA was 95% and ranged from 79 to 70% for T, A, and E_2.

Precision

Precision for androgens and E_2 was calculated by repeatedly analyzing 2 ml of male plasma (obtained from a local blood bank) on the same day and on different days. Results are reported in Table 1.

Validation of the equilibration method for 4-OHA

To validate the equilibration method for 4-OHA we compared two determinations of 4-OHA: the first one performed using the method described above and the second by adding, at the same time as the internal standard, a large excess of E_2 (E_2/4-OHA = 1000/1). This amount of E_2 was added in order to displace 4-OHA from binding protein. If the time for equilibration of the standards with proteins is insufficient the PAR of the sample with E_2 would be higher than that of the sample without E_2. The comparison was performed for two different concentrations of 4-OHA using two samples of the formulation of 8.0 μm particle size: the first at high 4-OHA concentration (volunteer 1 sample at 24 h) and the second at low concentration (volunteer 1 sample at 96 h). Every determination was performed in triplicate. Results are summarized in Table 2. There was no significant difference between the determinations made in the presence or absence of E_2. This experiment demonstrates that the 4-OHA determination method is not affected by errors due to 4-OHA binding to proteins.

Table 1. Precision of androgens and E_2 determination

Steroid	T (ng/ml)	A (ng/ml)	E_2 (pg/ml)
	Intraassay		
Mean	2.17	0.49	24.76
n	6	6	4
SD	0.059	0.02	1.95
CV%	2.7	4.1	7.8
	Interassay		
Mean	2.44	0.56	18.25
n	6	5	5
SD	0.212	0.11	5.3
CV%	8.7	7	15

Table 2. PAR in the same samples pre-incubated with E_2 and treated according to the standard procedure (see text for details)

Concentration	Sample (PAR)	Sample + E_2(PAR)
Low		
Meas. 1	0.138	0.138
Meas. 2	0.136	0.130
Meas. 3	0.156	0.145
Mean	0.143	0.138
High		
Meas. 1	0.443	0.448
Meas. 2	0.436	0.457
Meas. 3	0.472	0.426
Mean	0.450	0.457

RESULTS

The values of 4-OHA determination for both formulations are summarized in Table 3 and Fig. 1, respectively. The two profiles were analyzed using a three exponential model (SIPHAR ver.4, Simed; Creteille, France). For the 4.2 μm formulation the three half-life values were $t_{1/2\alpha} = 7.35$ h; $t_{1/2\beta} = 15.30$ h and $t_{1/2\gamma} = 57.33$ h. A.U.C. calculated between 0 and 336 h was 1135.2 ng/ml/h, the total body clearance was 0.22 l/h. C_{max} and t_{max} were 22.0 ng/ml and 27.6 h, respectively. Values for the same parameters with the 8.0 μm formulation were: $t_{1/2\alpha} = 6.80$ h; $t_{1/2\beta} = 13.53$ h and $t_{1/2\gamma} = 85.66$ h; A.U.C. (0–336 h) = 978.30 ng/ml/h; total body clearance 0.24 l/h. C_{max} and t_{max} were 13.5 ng/ml and 38 h, respectively.

The comparison between the two formulations was performed according to the Wilcoxon test for paired data. Even though the formulation with 4.2 μm particle size provided the higher 4-OHA plasma levels during the 48 h following the injection (the A.U.C. values between 0 and 48 h were 744.1 and 448.08 ng/ml/h for the 4.2 and the 8.0 μm formulations, respectively), these values were not significatively different as were all the other kinetic parameters. With the formulation with 4.2 μm particle size we studied the effects of 4-OHA using samples collected 0, 24, 48, 96 and 240 h after drug injection. Figure 2 shows E_2 and androgen mean plasma profiles. Table 4 summarizes results for E_2. A 250 mg dose of 4-OHA produced a 50% reduction in E_2 levels at 24 h in all subjects except subject 2. This decrease was statistically significant according to the Wilcoxon test for paired data ($P = 0.035$). No significant correlation between 4-OHA and E_2 plasma levels at 24 h was found ($r = 0.44$). E_2 levels returned to the basal levels after 1 week. No significant changes in androgen levels were

Table 3. 4-OHA values (ng/ml) for the two formulations

Time h	0	4	8	12	24	30	36	48	54	60	72	96	168	240	336
Particle size: 4.2 μm															
Vol. 1	0.0	20.3	21.7	12.3	20.6	22.9	27.9	9.8	6.4	4.8	2.9	1.5	0.2	0.1	—
Vol. 2	0.0	19.1	13.8	20.3	22.8	23.6	15.4	11.2	6.1	4.4	3.7	1.2	0.2	—	—
Vol. 3	0.0	20.6	20.1	19.7	30.6	26.4	17.8	12.6	9.3	6.7	4.8	1.5	0.3	0.2	—
Vol. 4	0.0	11.6	9.2	10.0	10.1	12.1	12.9	12.1	9.3	4.8	5.5	3.0	1.1	0.3	0.2
Vol. 5	0.0	7.3	11.5	16.1	22.9	23.7	26.1	14.4	6.5	7.6	3.1	1.5	0.3	1.1	—
Vol. 6	0.0	10.8	9.6	7.8	7.4	8.6	6.5	6.7	5.2	3.2	4.3	3.2	2.1	1.3	1.1
Mean	0.0	15.0	14.3	14.3	19.1	19.5	17.8	11.1	7.1	5.2	4.0	1.9	0.7	0.5	0.2
SD	0.0	5.7	5.4	5.1	8.7	7.3	8.1	2.6	1.7	1.6	1.0	0.9	0.8	0.5	0.4
CV%	0.0	40.0	40.0	30.0	40.0	40.0	40.0	20.0	20.0	30.0	20.0	90.0	110.0	100.0	200.0
Particle size: 8.0 μm															
Vol. 1	0.0	16.0	16.1	7.8	14.7	11.5	9.6	11.3	8.5	5.9	4.0	1.8	0.5	0.3	—
Vol. 2	0.0	4.1	7.7	7.2	5.5	5.1	6.9	9.4	6.0	6.7	5.9	4.0	1.7	1.0	0.8
Vol. 3	0.0	5.8	4.2	3.8	5.8	4.7	5.6	8.9	5.5	6.3	4.8	3.4	1.6	1.3	1.0
Vol. 4	0.0	7.4	11.4	7.9	17.7	14.9	6.9	4.7	4.2	2.9	2.6	1.5	1.2	0.8	0.6
Vol. 5	0.0	8.2	10.5	13.6	11.5	13.8	13.5	16.7	14.0	11.3	8.9	3.8	1.5	0.3	—
Vol. 6	0.0	11.4	11.4	12.2	9.8	8.9	13.6	10.1	7.1	6.1	4.5	2.7	0.9	0.4	—
Mean	0.0	8.8	10.2	8.8	10.8	9.8	9.4	10.2	7.6	6.5	5.1	2.9	1.2	0.7	0.4
SD	0.0	4.3	4.0	3.6	4.8	4.3	3.5	3.9	3.4	2.7	2.1	1.0	0.5	0.4	0.4
CV%	0.0	50.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	30.0	40.0	60.0	1.0

Measures below method sensitivity limit (10 pg/ml) were pointed out with '—'; for statistical evaluation the above mentioned value was used.

observed. Side effects were not observed with either formulation.

DISCUSSION

Several relevant observations stem from this study. First, the particle size does not seem to play an important role in determining the kinetics of 4-OHA in human plasma. Second, after treatment with the formulation with 4.2 μm particle size, 4-OHA in a 250 mg dose produced only a partial suppression of blood E_2 levels which returned to basal levels after 1 week. Moreover, A and T did not show any significant change. Finally, the effect of 4-OHA on E_2 circulating levels seems to coincide with the

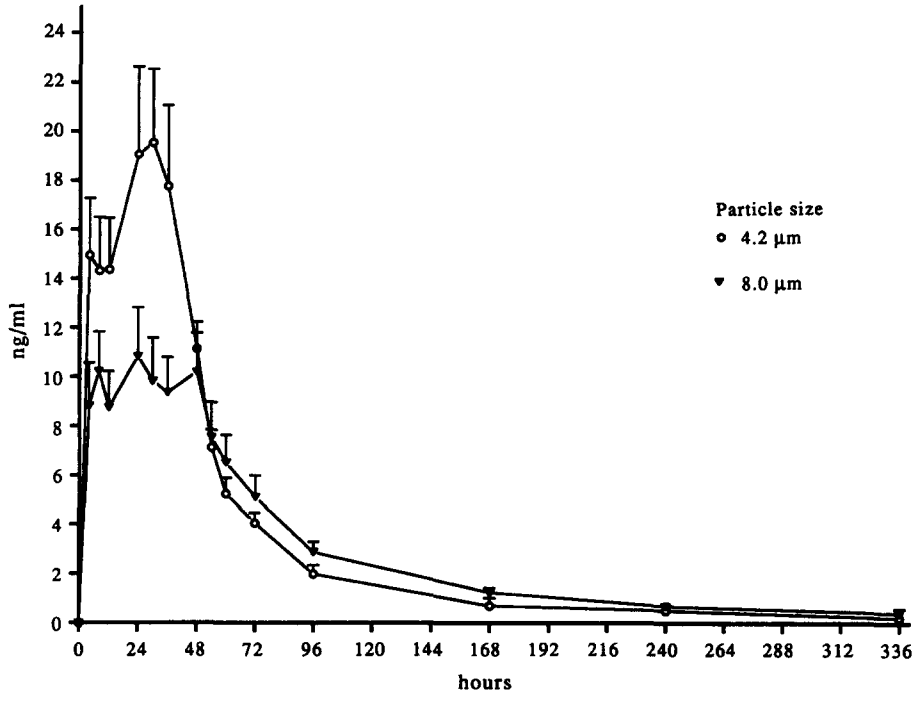
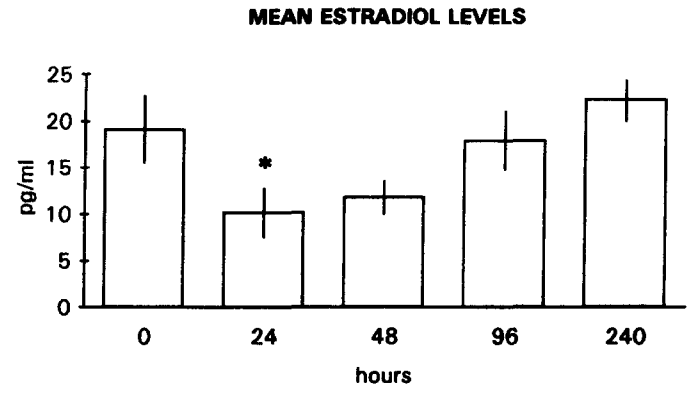
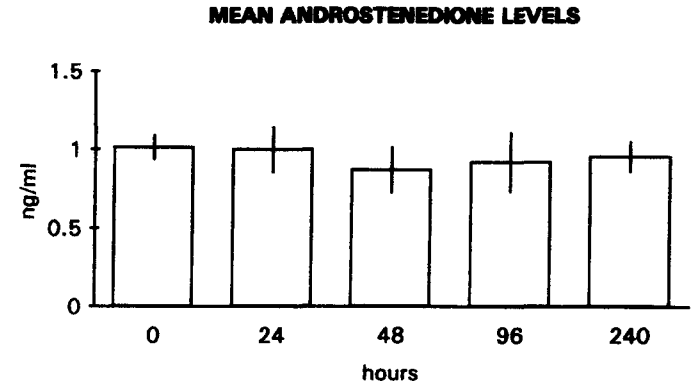


Fig. 1. Comparison between the mean pharmacokinetic profiles of the two formulations of 4-OHA differing in particle size (4.2 and 8.0 μm). Results are expressed as mean ± SE (ng/ml) of 6 subjects. Analyzed according the three exponential model (Siphar ver. 4) for calculations of pharmacokinetic parameters the difference between the two formulations was not statistically significant (Wilcoxon test for paired data).





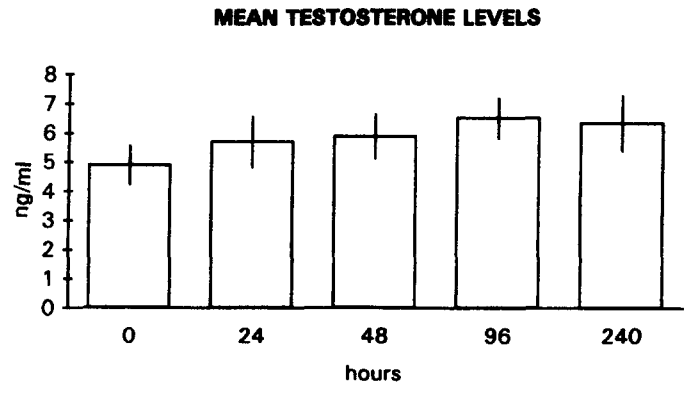


Fig. 2. Columns indicate the mean level and bars the standard error for the three steroids measured in blood. The analyses were performed on 6 subjects. The asterisk marks the data significantly different from the basal values according to the Wilcoxon test for paired data ($P = 0.035$).

Table 4. E_2 values (pg/ml) after a single 250 mg injection of 4-OHA (4.2 μm)

Time h	0	24	48	96	240	Reduction %
Vol. 1	16.83	—	—	15.64	20.16	64.30
Vol. 2	10.61	8.76	14.33	8.14	17.43	17.40
Vol. 3	20.17	8.76	11.66	20.91	20.11	56.50
Vol. 4	37.35	24.20	19.40	31.81	31.82	48.00
Vol. 5	14.25	—	10.63	10.94	25.75	57.80
Vol. 6	15.34	7.14*	8.69	19.73	18.25	53.40
Mean	19.09	10.14	11.78	17.86	22.25	49.70
SD	8.65	6.39	4.26	7.69	5.03	
CV%	45	63	36	43	23	

Measures below method sensitivity limit (6 pg/ml) were pointed out with '—'. 6 pg/ml were assigned to volunteers 1 and 5 for statistical evaluation. Reduction % was evaluated comparing the lowest observed value with the basal level for each volunteer. The '*' indicates the value significantly different from the basal value ($P = 0.035$) according to the Wilcoxon test for paired data.

peak of the maximum concentration of the inhibitor.

The reduction of E_2 levels (about 50%) observed after treatment with 4-OHA is similar to that reported by El Etreby *et al.* [10] in male volunteers using a different aromatase inhibitor with similar structure and mechanism of action. A possible explanation for this phenomenon could be the role played by other enzymatic activities involved in E_2 metabolism such as the sulfotransferase and sulfatase activities that are not affected by the drug [11]. This hypothesis is reinforced by the absence of correlation between E_2 suppression and 4-OHA circulating levels in our study. The role of sulfotransferase and sulfatase activities should be more relevant in acute experiments (such as those reported in the present paper) than during chronic administration when presumably the pool of estrogen sulfates is progressively decreased.

The recovery of the basal estrogen levels after 1 week found in our male subjects is again in agreement with El Etreby *et al.* [10]. On the other hand this phenomenon has not been observed in postmenopausal women treated with 4-OHA in whom estrogen levels remained low after 1 week [12]. Because the blood production rates of aromatizable androgens (A + T) in men are at least four times higher than those of postmenopausal women [13, 14], we can reasonably hypothesize that the quicker recovery of basal estrogen levels in men are due to the larger availability of aromatizable substrates.

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